The relative biological effectiveness of proton irradiation in dependence of DNA damage repair

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Abstract: Clinical parameters and empirical evidence are the primary determinants for current treatment planning in radiation oncology. Personalized medicine in radiation oncology is only at the very beginning to take the genetic background of a tumor entity into consideration to define an individual treatment regimen, the total dose or the combination with a specific anticancer agent. Likewise, stratification of patients towards proton radiotherapy is linked to its physical advantageous energy deposition at the tumor site with minimal healthy tissue being co-irradiated distal to the target volume. Hence, the fact that photon and proton irradiation also induce different qualities of DNA damages, which require differential DNA damage repair mechanisms has been completely neglected so far. These subtle differences could be efficiently exploited in a personalized treatment approach and could be integrated into personalized treatment planning. A differential requirement of the two major DNA double-strand break repair pathways, homologous recombination and non-homologous end joining, was recently identified in response to proton and photon irradiation, respectively, and subsequently influence the mode of ionizing radiation-induced cell death and susceptibility of tumor cells with defects in DNA repair machineries to either quality of ionizing radiation. This review focuses on the differential DNA-damage responses and subsequent biological processes induced by photon and proton irradiation in dependence of the genetic background and discusses their impact on the unicellular level and in the tumor microenvironment and their implications for combined treatment modalities.

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PROTON THERAPY SPECIAL FEATURE: REVIEW ARTICLE

The relative biological effectiveness of proton irradiation in dependence of DNA damage repair

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ABSTRACT

Clinical parameters and empirical evidence are the primary determinants for current treatment planning in radiation oncology. Personalized medicine in radiation oncology is only at the very beginning to take the genetic background of a tumor entity into consideration to define an individual treatment regimen, the total dose or the combination with a specific anticancer agent. Likewise, stratification of patients towards proton radiotherapy is linked to its physical advantageous energy deposition at the tumor site with minimal healthy tissue being co-irradiated distal to the target volume. Hence, the fact that photon and proton irradiation also induce different qualities of DNA damages, which require differential DNA damage repair mechanisms has been completely neglected so far. These subtle differences could be efficiently exploited in a personalized treatment approach and could be integrated into personalized treatment planning. A differential requirement of the two major DNA double-strand break repair pathways, homologous recombination and non-homologous end joining, was recently identified in response to proton and photon irradiation, respectively, and subsequently influence the mode of ionizing radiation-induced cell death and susceptibility of tumor cells with defects in DNA repair machineries to either quality of ionizing radiation.

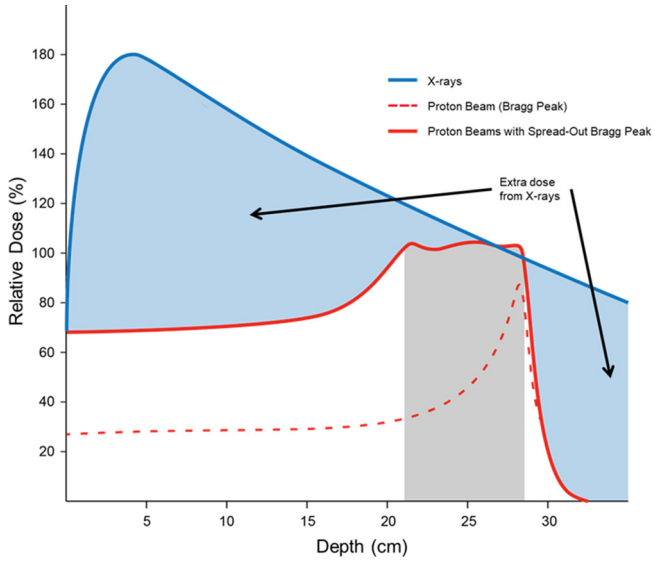
This review focuses on the differential DNA-damage responses and subsequent biological processes induced by photon and proton irradiation in dependence of the genetic background and discusses their impact on the unicellular level and in the tumor microenvironment and their implications for combined treatment modalities.

INTRODUCTION

Radiotherapy alone or in multimodality approaches is applied in 45–60% of all cancer patients, but despite technical innovations approximately only 50% are cured ([1] and references therein). At present, the most commonly used mode of radiotherapy with high energy linear accelerators is using an externally generated photon beam directed towards the exact delineated tumor site. Other forms of radiation include radiotherapy with charged particles such as electron beams, protons and heavier charged ions such as ^{12}C. Of these, proton radiotherapy is becoming a reasonable alternative worldwide.[2–4] Stratification towards a specific quality of ionizing radiation is primarily based on clinical parameters, not taking any biological aspects into consideration.

The major difference between photon- and particular proton-based radiotherapy is the spatial distribution of energy deposition. Photon beams have the highest dose deposition close to the entrance surface and continuously deposit dose at the whole path throughout the tissue. Generally, this involves healthy tissue being co-irradiated proximal and distal to the target volume. In contrast, proton beams commonly deposit a lower dose in the entry field, and maximum dose deposition occurs within the so-called Bragg peak at a depth defined by the velocity of the applied protons. Behind this Bragg peak region—or spread-out Bragg peak (SOBP) in clinical applications—no significant dose is deposited[5] (Figure 1). Thereby, a reduced exposure of dose-limiting organs-at-risk (OARs), *e.g.* the brain stem, the optical nerve and the oral cavity, to low and intermediate doses of ionizing radiation is achieved by proton radiotherapy.[6,7] Eventually, this will result in a reduced risk of normal tissue toxicities and secondary malignancies in these co-irradiated organs close to treated entities such as skull-based and intracranial tumors, uveal melanoma and head and neck tumors, respectively.[8–10]

Figure 1. Differential depth dose distributions of photons versus protons. Photon beams have the highest dose deposition close to the entrance surface and continuously deposit dose at the whole path throughout the tissue. In contrast, proton beams deposit a lower dose in the entry field, and maximum dose deposition occurs within the so-called Bragg peak or SOBP in clinical applications. The reduced volume of healthy tissue exposed to intermediate and low doses of proton radiotherapy results in a reduced co-irradiation of dose-limiting OAR. OARs, organs-at-risk; SOBP, spread-out Bragg peak.



Progressive cognitive decline and impaired brain development represent major risk factors after conventional cranial radiotherapy in pediatric patients. Thus, particle radiotherapy represents an ideal treatment modality to reduce these side-effects in the central nervous system in pediatric tumor patients.[11,12]

Preclinical *in vitro* and *in vivo* experiments suggest an enhanced potency for proton- *vs* photon-irradiation. This enhanced relative biological effectiveness (RBE) is accounted for by the generic RBE value of 1.1 used in the clinics. In general, the RBE depends on the linear energy transfer (LET), the radiation dose, the number of fractions applied, the dose range and the biological system or end point analyzed.

The RBE is the ratio of the dose of high-energy photons, *e.g.* 60Co γ-rays or linear accelerator generated X-rays, relative to that of protons required to produce the same biological response. This effect is generally considered to be relatively small for protons, and a generic RBE of 1.1 has been used throughout its history for dose specification with virtually no exceptions being made for the dose/fraction, position in the SOBP, initial beam energy, or the tissue being irradiated. The global use of an RBE value of 1.1, *i.e.* a 10% higher biologic effectiveness of protons compared to photons, is based primarily on radiobiology experiments conducted in the 70's and 80's.[13] However, the LET varies along a clinically relevant SOBP. For example, in case of a 62 MeV proton beam with a 10 mm SOBP centered at 25 mm depth, the LET ranges from approximately 1 keV/μm at the entrance field, to 4 keV in the SOBP and reaches up to 25 keV/μm at the Bragg Peak.

Eventually, several groups also demonstrated a varying RBE depending on the position cells and tissue were placed within the SOBP, with the highest RBE when cells were positioned in the Bragg peak area.[14,15] This corresponds to enhanced cell killing per gray of irradiation as LET increases. These considerations result in "LET painting" as an approach to shift distal end, high LET and thus high RBE irradiation away from critical organs into the tumor treatment volume.[16–18] However, the clinical decision at the leading proton facility, the Harvard Cyclotron Laboratory, was made to proceed with a RBE factor of 1.1 as the basis of treating patients.[19] Subsequent clinical data of the last 20–30 years have though confirmed the usefulness of the factor of 1.1 in clinical practice.

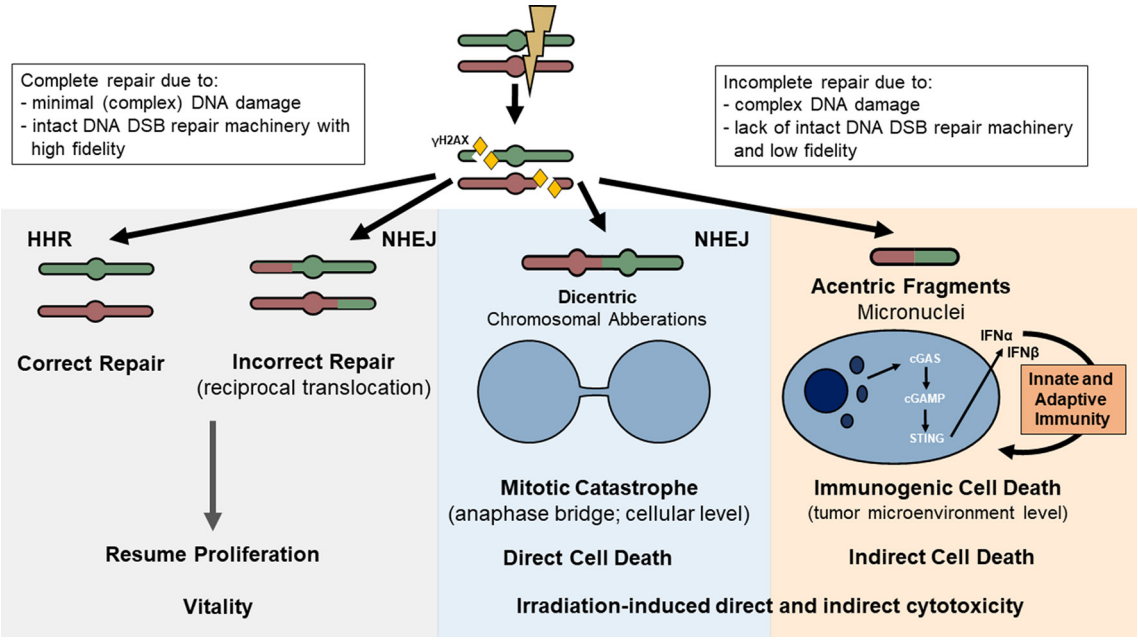
Based on the improved experimental systems, the increased knowledge gained during the last decades on ionizing radiation-induced biological responses and the increasing amount of proton radiotherapy centers with integrated radiobiological research facilities, molecular and cellular-oriented studies are now routinely performed to investigate differential stress responses and differential damage profiles induced by proton *vs* photon irradiation. Thereby, the RBE will be characterized not only in relation to the differential energies but also from the differential processes induced on the molecular, cellular and (patho-) physiological level. These putative differences could eventually be important for different treatment strategies (*e.g.* as part of combined treatment modalities) and for patient stratification, challenge the use of a generic RBE and asks to integrate biological parameters into treatment planning.[20,21]

Recent insights from our own and other research laboratories indicate that differential qualities of DNA damage and subsequently differential DNA-damage responses (DDR) are induced in response to photon and proton irradiation. These differential "biologies" may be important for the concept of personalized medicine in radiotherapy. Nowadays, treatment planning of an individual patient primarily takes clinical and physical parameters into account. Eventually, stratification towards proton *vs* photon irradiation might also take these differential "biologies" on the personalized level into consideration.

Ionizing radiation induced cytotoxicity on the individually targeted cell

Ionizing radiation induces DNA double-strand breaks (DNA DSB), which represent the major cytotoxic insult. These DSBs are to a large extent correctly repaired by the two major DNA DSB repair pathways, namely the error-free homologous recombination repair (HRR) and the more error-prone non-homologous end joining (NHEJ) process.[22] NHEJ is quantitatively highly efficient to repair most of the DSBs throughout the cell. However, the quality of repair steadily decreases with increasing amounts of DNA DSBs and NHEJ-mediated chromosomal translocations might even lead to toxic chromosomal aberrations.[23] In an initial step, Ku70/80 heterodimers are formed at the broken end, which leads to the recruitment of DNA-PKcs, followed by processing of the "dirty" ends by specific DNA nucleases and polymerases and religation of the broken strands by the XRCC4-DNA Ligase IV complex.

Figure 2. Ionizing radiation induced cytotoxicity on the individually targeted cell level and via the tumor microenvironment. Incorrectly repaired DNA double strand breaks might lead to chromosomes containing reciprocal translocations of chromosomal regions. These monocentric chromosomes can still be segregated into the two surviving daughter cells following mitosis. On the other hand, most cells with dicentric chromosomes (shown) will undergo cell death. These chromosomal aberrations activate a mitotic checkpoint and will eventually undergo mitotic catastrophe in the initial or in one of the following cell cycles, respectively (direct cytotoxicity). Acentric DNA fragments (shown) are at the same time encapsulated into micronuclei, which are often leaky, and subsequently release small DNA fragments into the cytosol. These small DNA fragments stimulate the expression and secretion of imunostimulatory cytokines via the cGAS-STING pathway, and are now regarded as co-factors and starting point for ionizing radiation-induced immunogenic cell death. Immunogenic cell death contributes to tumor control acting also against tumor cells (indirect cytotoxicity), which initially survived direct targeting by ionizing radiation.



In contrast to NHEJ, HRR is an error-free process. Following MRN- and CtIP-dependent 3`−5`DNA resection, the single-stranded DNA overhangs are stabilized by the heterotrimeric ssDNA-binding complex RPA.[24] With the help of the tumor suppressor BRAC2, RPA is then replaced by RAD51 to form RAD51 nucleoprotein filaments and Holliday junctions (HJ) with complementary sequences on the adjacent sister chromatid. Eventually, these structures are resolved by several HJ processing factors on completion of DNA polymerase-dependent error-free rewriting of the damaged DNA sites.[25–27]

Incorrectly repaired DNA DSB might lead to reciprocal translocations of chromosomal regions. These newly rearranged, duplicated but still monocentric chromosomes can still be segregated into the two surviving daughter cells following mitosis. On the other hand, most cells with dicentric chromosomes undergo cell death. These chromosomal aberrations are formed due to incorrect DNA DSB repair and form unresolvable anaphase bridges during chromosomal segregation in M-phase and activate a mitotic checkpoint. Cells with unresolved, prolonged mitotic checkpoints or cells with extended chromosomal aberrations, which managed to slip into the next cell cycle, will eventually undergo mitotic catastrophe in the initial or in one of the following cell cycles, respectively. Overall, these cells undergo cell death as a direct consequence of ionizing radiation-induced DNA damage, incomplete DNA damage repair and the formation of chromosomal aberrations, which can not be properly segregated into the two daughter cells (Figure 2).

Cellular hypersensitivity towards proton irradiation in dependence of the genetic background

As part of an RBE-oriented non-small cell lung cancer (NSCLC) cell line screen, hypersensitivity to proton irradiation could be linked to defects in a specific DNA damage repair machinery.[28] Subsequently, the research group of H. Willers (Massachusetts General Hospital, Boston) could identify specific alterations of the Fanconi-Anemia (FA)/BRCA- replication-coupled HRR pathway of DNA repair in three out of 17 cell lines with an increased RBE.[28] For these comparative experiments, lung cancer cell lines were positioned at the mid-SOBP of a clinical proton beam (235 MeV). Control experiments with genetically modified lung cancer cells validated the relevance of this pathway, and supported the hypothesis of specific proton hypersensitivity due to HRR-defects (RBE-shift from 1.1 to 1.39 in dependence of FANCD2-expression level). The same group performed additional control experiments on two downstream elements of the FA/BRCA-pathway, namely Slx4(FancP) and Mus81.[29] Slx4- and Mus81-deficient cells were also hypersensitive towards proton irradiation in comparison to their isogenic wildtype counterpart cells (RBE-shift from 1.1 to 1.29 in dependence of Mus81-mutational status).

In parallel to this genomic profiling approach, hypersensitivity towards proton irradiation in HRR-deficient cells has been corroborated in several established cancer cell lines. PEO1-ovarian carcinoma cells that are lacking intact BRCA2-expression but are otherwise genetically identical to PEO4-ovarian carcinoma cells, were hypersensitive towards proton in comparison to photon irradiation. Likewise, clonogenic survival of A549 NSCLC cells that were depleted of Rad51, was strongly reduced in response to proton irradiation in comparison to control cells that were irradiated with either quality of ionizing radiation.[30] Downregulation of Rad51 also resulted in delayed γH2AX-foci repair kinetics (see below). These experiments were also performed with cells positioned in the middle of the SOBP and with a clinical proton beam. Of note, besides HHR and NHEJ as major DNA DSB repair machineries, additional DNA DSB damage repair pathways exist, which also co-determine the cellular response to ionizing radiation. However, at this stage and due to the lack of defined comparative experiments, we cannot estimate, *e.g.* the impact of alternative NHEJ (Alt-NHEJ) to a putative shift of the RBE and thus a differential radiation response to proton *vs* photon radiation.

A set of well-characterized chinese hamster ovary (CHO) cell lines was traditionally used in radiobiology to define the involvement of the different DNA DSB repair machineries for photon irradiation-induced DNA damage. These CHO cell lines have defined defects in different DNA repair machineries and are now also very suitable to characterize subtles differences in the cellular response to different qualities of ionizing radiation.[31–33]

In comparison to the corresponding wildtype cells, an increased RBE was determined in the HRR-defective cells, indicating hypersensitivity of HRR-deficient cells towards proton irradiation ($RBE_{37\%}$: 1.54 ± 0.10 and $RBE_{10\%}$: 1.44 ± 0.06 HRR-deficient cells *vs* $RBE_{37\%}$: 1.25 ± 0.05 and $RBE_{10\%}$: 1.29 ± 0.04 wildtype cells).The RBE (survival fraction as end point) was determined with cell positioned in the middle of the SOBP, with a length of 5 cm and maximum proton energy of 138 MeV. NHEJ-defective cells (XR-C1; DNA-PKcs-deficient CHO cells) were overall more sensitive to both types of ionizing radiation in comparison to wildtype cells. However, no hypersensitivity towards proton irradiation was identified in the NHEJ-defective cells.[34] Of note, differential patterns of chromosomal aberrations, in particular with an increased amount of smaller fragments, were identified in response to proton and photon irradiation (see below).

The HRR status of tumors predicts treatment sensitivity to several anticancer agents such as cisplatinum and taxanes. Furthermore, the concept of synthetic lethality was developed based on the identification of BRCA1/BRCA2-mutations leading to enhanced sensitivity to PARP-inhibitors.[35] Large-scale next generation sequencing studies on molecular profiling of solid tumors beyond BRCA1/2 suggest that more than 15% carry mutations in HRR.[36] Based on the enhanced sensitivity of tumor cells with HRR-mutations to proton irradiation and preference of proton-induced DNA damage to be repaired by HRR, stratification of patients towards proton *vs* photon radiotherapy should also take the mutational status of the tumor into consideration.

Damages at the molecular DNA level and DNA damage-related signaling should be determined to detect and quantify ionizing radiation-induced DSBs and repair of DNA DSBs over time. However, for the sake of convenience, most studies only focus on the formation and processing of γH2AX-foci in response to ionizing radiation, which are easily detectable. As part of the initial DNA damage response to DSB, the histone variant H2AX are posttranslationally phosphorylated in order to mark the site of the DNA DSB and to initiate DNA DSB repair. γH2AX-foci detection has now become a powerful method to quantify DNA DSBs induced by ionizing radiation. Phosphorylated H2AX was originally named γH2AX as it was first observed in γ-ray-exposed cells.[37] Clusters of γH2AX molecules at the site of DNA breaks allow detection and quantification of individual DSBs by using γH2AX-oriented antibodies.[38] Interestingly, the specific shape and the kinetics of γH2AX-foci formation and processing (incl. 53BP1-foci, which also accumulate at the site of DNA damage) might be used to mark repair-defective and proton irradiation hypersensitive tumors. Using these foci as biomarkers was suggested by the research group of H. Willers, who identified an increased size of 53 BP-1 foci in FA/BRCA-pathway-deficient tumor cells, in particular in response to proton irradiation. These large foci might be due to an increased complexity of clustered DNA damage in response to proton irradiation.[28,39] Since the FA/BRCA-pathway is specifically involved in replication fork maintenance and repair, increase of foci sizes might result from impaired repair of replication forks that collide with clustered proton damages.

In the genetically defined CHO cell system no significant quantitative differences in the initial amount of DNA DSBs were observed in cells irradiated by the two types of irradiation. However, elevated numbers of residual γH2AX-foci were detected in the HR-deficient cells after proton irradiation, indicative for delayed repair kinetics after proton irradiation. Cells were always irradiated in the middle of the SOBP in order avoid additional LET-depending influences across the SOBP. These results support the notion of a differential quality of DNA damage induced by proton *vs* photon irradiation. Furthermore, the qualitative difference on the level of the DNA damage response correlate with the quantitative clonogenic cell survival data and indicate a specific requirement for FA/HRR for potent DNA DSB repair in response to proton irradiation.

Recently, DNA DSB repair kinetics were investigated in response to photon and proton irradiation analyzing formation and removal of γH2AX and 53BP1-foci in murine prostate adenocarcinoma and murine embryonic fibroblast.[40] In this study, cells were exposed to the same dose but irradiated at different positions with the proton beam (plateau and Bragg Peak). A 105.5 MeV proton beam, was decelerated by a range shifter to approximately 31 MeV within the Bragg-peak. For the plateau proton set-up, a higher energy of 220 MeV was used to ensure that the proton beam was still in the plateau zone when reaching the cells after crossing the same range shifter. Consequently, the cells were hit by a proton beam with 187 MeV. Again, similar amounts of foci were induced in response to photon and proton irradiation, but foci-induction, processing and removal was delayed in cells

irradiated in the Bragg peak of the proton beam. Furthermore, the shape of Bragg peak-induced foci was more irregular and larger than foci induced by photon and plateau-protons. These differential characteristics might be linked to overlapping DNA lesions in close proximity to another, also resulting in more complex, clustered lesions. Such lesions were as also observed on irradiation with high LET charged particles, which also require more time for repair and eventually differential DNA repair machineries.[41,42] An increased complexity of proton-induced DNA damage with more persistent foci but only at the distal end of a SOBP was also demonstrated in detailed comparative irradiation studies with cells positioned along a modulated SOBP proton beam[43] confirming the overall low-LET-quality of proton exposure but with increased LET towards the distal end. Again, these qualitative DNA-damage-oriented results on the unicellular level correlate with differential RBEs (clonogenic cell survival) when cells are irradiated at different positions within a SOBP *in vitro*[44,45] and *in vivo*.[14]

Overall, these preclinical results link a putative differential quality of DNA damage in response to proton *vs* photon irradiation to a hypersensitivity to proton irradiation and increased cytotoxicity on the unicellular level.

Hypersensitivity towards proton irradiation in the tumor microenvironment

Ionizing radiation may induce different modes of cell death, which are primarily linked to the origin of the targeted cells and their differentiation status. Besides mitotic catastrophe (see above), also enhanced cell apoptosis was identified after low LET proton irradiation in comparison to photon irradiation.[46] Recently, the irradiation-induced response of the immune system and its related induction of immunogenic cell death (ICD) has gained of interest, and—even though still to be proven—proton and photon-irradiation might induce different levels of ICD, which again might be linked to the status of the two major DNA DSB repair machineries.

As indicated above, acentric fragments have long been regarded as waste by-products of chromosomal aberrations and less relevant for the direct cytotoxic potency of ionizing radiation. These small acentric fragments are encapsulated into micronuclei (MN) and these MN are actually used as biomarkers for the induction of mitotic catastrophe. Interestingly though, these MNs and the encapsulated small DNA fragments are now regarded as starting point of co-stimulatory factors for ionizing radiation-induced ICD.

Irradiation activates an anti tumor immune response through the release of danger signals and inflammatory cytokines thereby promoting dendritic cells to cross-present released antigens to T cells.[47] A thereby triggered cytotoxic T cell response might be directed toward the primary irradiated tumor, but might also acts against non-irradiated metastasis via an abscopal effect, directed against non-irradiated lesions. Interestingly though, the acentric DNA fragments, classified as by-products of the incorrectly repaired chromosomes, act as initial co-factors for the activation of this so called cancer-immunity cycle in response to irradiation.

While compartmentalization of the cell separates nuclear-located chromosomes from the cytosol and prevents sensing of DNA in the cytosol, rupture of micronuclei gives access of cytosolic proteins to DNA. Likewise, small DNA fragments may also leak out from MN into the cytosol. DNA fragments trigger the formation of the second messenger cGMP-AMP (cGAMP) by the cytoplasmic protein GMP-AMP synthase (cGAS), and cGAMP activates the Stimulator of Interferon Genes (STING)-dependent signal transduction cascade leading to the expression and secretion of pro-inflammatory cytokines such as interferone α and β[48,49]. These cytokines contribute to tumor recruitment and activation of Batf3-lineage dendritic cells, which are responsible for correct priming of T-cells as part of the cancer immunity cycle[47] (Figure 2).

As such, incorrectly repaired ionizing radiation-induced DNA damage results in DNA-insults (dicentric chromosomes) directly acting in a cytotoxic way on the unicellular level and may indirectly (acentric fragments) stimulate an immune-mediated tumor-oriented toxic microenvironment. Both mechanisms are induced in response to different qualities of ionizing radiation, but to different extents and in dependence of different genetic backgrounds.

A few studies have so far directly compared the regulation of immune-stimulatory factors and surface molecules in response to different qualities of ionizing radiation that are required for successful ionizing radiation-triggered ICD. A common signature of surface molecules involved in immune recognition, tumor-associated antigens and calreticulin surface expression was observed in multiple tumor cell lines in response to photon and proton irradiation, relevant for successful cytotoxic T-lymphocyte mediated cell killing.[50] On the other hand, inflammatory factors such as IL6, IL8 and CXCL12 were generally less extensively upregulated by proton irradiation in comparison to photon irradiation.[51] Of interest, proton irradiation in particular at higher dosages results in increased rates of micronuclei formation in comparison to photon irradiation as characterized in thyroid follicular cells (0–12 Gy) and human peripheral blood lymphocytes (2–4 Gy).[46,52] Since micronuclei encapsulate small DNA fragments, an enhanced rate of micronuclei formation may also correlate with a higher rate of acentric and small DNA fragments generated in response to proton irradiation.[34]

The generation of small DNA fragments in response to irradiation is also determined by an intact DNA damage response. Interestingly, micronuclei formation and cGAS-STING activation was specifically increased in a BRCA2- and thus HR-deficient background in tumor cells as part of replicative stress even in absence of irradiation-induced DNA damage.[53] Given the hypersensitivity of HR-defective tumor cells to proton irradiation on the unicellular level, it will be of interest to determine activation of the cancer immunity cycle and induction of ICD in tumors derived from HR-intact and HR-defective tumor cells in response to proton irradiation but in an otherwise intact tumor microenvironment.

Combined treatment modalities with HRR-interfering agents

A combined treatment modality with specific inhibitors of HRR could also lead to enhanced sensitivity towards proton versus photon irradiation. However, the development of pharmacological agents directly targeting moieties of the HRR machinery has been largely unsuccessful so far. Nevertheless, several chemotherapeutic agents exist, which eventually affect HRR, *e.g.* by the downregulation of respective HRR-elements. For example, low dose exposure of lung adenocarcinoma cells to the histone deacetylase inhibitor SAHA (Vorinostat) downregulates Rad51 protein levels but not critical elements of NHEJ, and thereby specifically reduces HRR activity.[54] Of note, DNA repair in SAHA-pretreated cells was in particular delayed after proton irradiation and much less so in response to photon irradiation, and cellular pretreatment with SAHA also translated into enhanced radiosensitivity towards proton irradiation.[30]

Interestingly, SAHA-based radiosensitization was also investigated in response to different qualities of ionizing radiation (photon, low LET proton and high LET carbon irradiation) in lung carcinoma cells versus normal human fibroblasts.[55] SAHA specifically sensitized tumor cells and less so normal fibroblasts, which is of interest towards an enhanced therapeutic window. Furthermore, these studies also demonstrated delayed DNA repair and most potent SAHA-based sensitization at low doses for proton irradiation.

Besides SAHA, other pharmaceutical agents in early developmental stage exist, which directly or indirectly reduce HRR-activity. The inhibitor of the heat shock chaperone hsp90 ganetespib counteracts irradiation-enhanced RAD51protein levels and thereby sensitizes lung adenocarcinoma cells for ionizing radiation.[56] The Brc-abl tyrosine kinase inhibitor gleevec, which is used to treat chronic myelogenous leukemia (CML), also reduces RAD51protein levels and thereby sensitizes for irradiation.[57] Likewise, reduced formation of the RAD51-BRCA2 complex is also induced on cellular treatment with inhibitors of the hepatocyte growth factor receptor Mesenchymal-Epithelial Transition (MET). MET is overexpressed in multiple types of human tumors and inhibition of MET kinase activity also sensitizes for ionizing radiation.[58]

Selective targeting of HRR even represents the mechanism of action for clinically applied chemotherapeutic agents and radiosensitizers, *e.g.* gemcitabine, when used in combination with photon radiotherapy.[59] Thus, a reduced dose of proton radiation might be sufficient when combined with HRR-co-targeting agents to achieve the same treatment outcome as when combined with classic radiotherapy.

On the other hand, inhibitors of NHEJ might sensitize more for photon than for proton irradiation. Indeed, the DNA-PKcs inhibitor NU7026 strongly delayed repair of photon- but not proton-induced DNA DSB damage and subsequently sensitized lung carcinoma and glioblastoma to a higher extent to photon than to proton irradiation.[30] These results also indicate that HRR and not NHEJ is the primary repair machinery for proton-induced DNA DSBs.

The identification of immunosuppressive mechanisms and respective blocking immune checkpoint inhibitors boosted an immense level of basic, translational and clinical research at the interface of radiotherapy and immunology leading to promising clinical trials of radiotherapy in combination with immune checkpoint inhibitors.[60] Radiotherapy has long been classified as immunosuppressive. However, preclinical studies with hypofractionated regimens have revealed that increased doses of ionizing radiation induce potent anti tumor immune responses, and several strategies have been developed to heat-up the cancer immunity cycle and to increase irradiation-induced immunogenic cell death.[61] Likewise, we also start to identify mechanisms on the molecular level how DNA damage drives the regulation of immune checkpoints.[62,63] In order to follow this line of research there is a great need to explore how the different qualities of ionizing radiation interfere with immunogenic cell death and the cancer immunity cycle. For example, TREX1 is an endonuclease, degrades small DNA fragments and thereby downregulates irradiation-induced immunogenic cell death (see above). TREX1 is upregulated in particular in response to single high doses of ionizing radiation, and thus, TREX1 inhibitors could be of great interest to stimulate immunogenic cell death in particular as part of a hypofractionated treatment regimen.[64] However, due to the different qualities of proton irradiation-induced DNA damage and a differential requirement for HRR as major DNA repair mechanism, the expression of TREX1 might also be regulated in a differential way and thereby influence immunogenic cell death in a differential way.

Recently, a novel inhibitor of the HRR-upstream-situated serine/threonine-specific protein kinase ATR (AZD6738) was demonstrated to sensitize for ionizing radiation *in vitro* and *in vivo*.[65] Interestingly, AZD6738 in combination with irradiation not only reduced clonogenicity, but also increased the amount of irradiation-induced cellular micronuclei. Thus, the ATR and other HR-oriented inhibitors could thereby not only increase hypersensitivity on the unicellular level but even increase immunogenic cell death on the tumor level in response to proton irradiation.

However, most treatment combinations have only been investigated with classic photon irradiation. It will be now important to perform both efficacy- and mechanistic-oriented studies with these clinically relevant agents in combination with proton radiotherapy. Eventually, these investigations could lead to the integration of biological parameters for patient's stratification to either quality of ionizing radiation.

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